Impact of high-intensity ultrasound on the formation of lactulose and Maillard reaction glycoconjugates

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ABSTRACT

A study on the impact of high-intensity ultrasound (US) on the formation of lactulose during lactose isomerization and on the obtention of lysine-glucose glycoconjugates during Maillard reaction (MR) was carried out in basic and neutral media respectively. As compared to equivalent conventional heat treatments, a higher formation of furosine, as indicator of initial steps of MR, was observed together with more advance of the reaction in samples treated by US, this effect being more pronounced with the increase of US amplitude (50-70%) and temperature (25-40 °C). Regarding the effect of US on lactulose formation, in general, in a buffered system (pH 10.0), US treatment at 70% of amplitude and 60 °C increased the rate of lactose isomerization, higher values of lactulose, epilactose and galactose being observed in comparison to conventional heating. Therefore, the results presented in this work showed an acceleration of both reactions by US, indicating the usefulness of this procedure to promote the formation of functional ingredients.

*Keywords***:** high-intensity ultrasound; conventional heating treatment; Maillard reaction; glycoconjugates; furosine; lactose isomerization; lactulose

1. Introduction

As it is well-known, non-enzymatic browning, (Maillard reaction (MR), sugar isomerization and sugar degradation such as caramelization) is perhaps the most complex reaction in food chemistry due to the number of compounds able to participate through different pathways giving rise to a complex mixture of products. MR takes place between free amino groups from amino acids, peptides, or proteins and the carbonyl group of reducing sugars during food processing and storage (Olano & Martínez-Castro, 2004). When this reaction is at the advanced stages, nutritional changes attributed to the participation of essential amino acids such as lysine or reduction of protein digestibility can be produced together with the formation of toxic compounds (Corzo-Martínez, Lebrón-Aguilar, Villamiel, Quintanilla-López, & Moreno, 2009). However, at the initial steps, this reaction has been demonstrated to be a useful tool to deliberately obtain, under controlled conditions, glycated proteins with improved technological and biological functionality (Oliver, Melton, & Stanley, 2006).

Isomerization of carbohydrates is also other very important chemical reaction that can take place in processed foods. During the heat treatment of milk or solutions of lactose in basic media, lactose is first transformed into lactulose (4-O-β-D-galactopyranosyl-D-fructose) followed by the breakdown into galactose and isosaccharinic acids to finally form the precursors of brown compounds (Berg & van Boekel, 1994). Lactulose is sweeter and more soluble than lactose and has a wide range of applications in the food and medical industries. It is the first commercially available prebiotic and can also be used as laxative and for the treatment of portal systemic encephalopathy (Mendez & Olano, 1979; Strohmaier, 1998; Zokaee, Kaghazchi, Zare, & Soleimani, 2002).

On the other hand, ultrasound (US) technology has emerged as an alternative processing to conventional thermal approaches, probably due to the fact that US makes use of physical and chemical phenomena that are fundamentally different compared with those applied in conventional procedures. US is environmentally friendly and offers benefits in terms of productivity, selectivity with better processing time and enhanced quality (Chemat & Khan, 2011; Chandrapala, Zisu, Kentish, & Ashokkumar, 2012) and, moreover, has the potential to develop new products with a unique functionality (Soria & Villamiel, 2010).

US can be used to promote certain reactions (Contamine, Faid, Wilhelm, Berlan, & Delmas, 1994), the MR being one of the most interesting. The acceleration effect of US in the intermediate (formation of HMF, absorbance measured at 294 nm, A_{294}) and final stages (absorbance measured at 420 nm, A_{420}) of MR has been studied in basic pH model systems of glycin-glucose (Guan, Zhang, Yu, Wang, Xu, Wang, et al., 2011), bovine serum albumin-glucose (Shi, Sun, Yu, & Zhao, 2010) and model systems at neutral pH of β-lactoglobulin with different carbohydrates (Stanic-Vucinic, Prodic, Apostolovic, Nikolic, & Velickovic, 2013). Mu, Zhao, Yang, Zhao, Cui, and Zhao (2010) also observed an acceleration of graft reaction between soy protein isolate and gum acacia by US treatment. However, to the best of our knowledge, no studies have been focused on the effect of US on the initial steps of MR during which scarce structural modifications of proteins takes place.

With respect to the effect of US on carbohydrates, Brochette-Lemoine, Trombotto, Joannard, Descotes, Bouchu, and Queneau (2000) observed a sonocatalysis effect during the course of the oxidation of primary hydroxyl groups of sucrose. It is also known the potential of US to modify the functional properties of carbohydrates (Panchev, Kirtchev, & Kratchanov, 1994; Seshadri, Weiss, Hulbert, & Mount, 2003; Sun, Hayakawa, & Izumori, 2004). Recently, Wang, Pan, Zhang, Sun, Fang, and Yu (2012) studied the combination of US

application and ionic liquid to enhance the enzymatic isomerization of glucose to fructose. In spite of these works, no investigation has been done on the effect of US on the isomerization of lactose, as a tool to accelerate the formation of lactulose.

Therefore, the objective of this work was to study the impact of high-intensity US on: i) the initial stages of MR in a model system of lysine-glucose (Lys-Glu) at neutral pH, and ii) the formation and degradation of lactulose during the isomerization of lactose in basic media.

2. Materials and methods

2.1. Chemicals

L-Lysine (Lys), glucose (Glc), lactose (Lac), lactulose (Lu), epilactose, galactose (Gal), phenyl-β-D-glucoside, and trimethylsilylimidazol were purchased from Sigma-Aldrich (St. Louis, MO). Sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate dihydrate, sodium hydrogen carbonate, anhydrous sodium carbonate, sodium hydroxide and potassium chloride were provided by Merck (Darmstadt, Germany). Hydrochloric acid was obtained from Panreac (Barcelona, Spain), HPLC grade acetic acid from Scharlau Chemie (Barcelona, Spain), and the commercial pure standard of furosine (ε-2-furoylmethyl-lysine) from Neosystem Laboratories (Strasbourg, France).

2.2. Preparation of Lysine-Glucose model systems and lactose solutions

Model systems were prepared dissolving glucose and lysine in 0.2 M sodium phosphate buffer (pH 7.0) to give equimolar solutions (1 M concentration of each reactant).

Lactose solutions were prepared by dissolving lactose at 10% concentration (w/v) in 8 mM sodium hydroxide solutions (initial pH values of 10.6) or 0.2 M sodium carbonate-bicarbonate buffer (pH 10.0).

Measurements of pH were carried out in all samples before and after treatments with a pH meter MicropH2001 (Crison Instruments, Barcelona, Spain) (data not shown).

2.3. Ultrasound and conventional heat treatments

Lys-Glc mixture and lactose solution (50 mL) were heated in a 250-mL Pyrex beaker in a water bath up to 25 or 40 °C, in the case of Lys-Glc model systems, or 60 °C for lactose solutions. At the end of heating ramp (6 and 8 minutes up to 40 and 60 °C respectively, identified as time 0 min), 2 mL-aliquots were taken and considered as starting point to compare both type of treatments (US and conventional). Upon reached the temperature tested, two sets of duplicate experiments were carried out: (i) in water bath (conventional treatment, CT) and (ii) with an ultrasonic probe (ultrasound treatment, UST).

(i) Conventional: samples remained in the water bath for 15, 30 and 60 min. Then, samples were immediately cooled in an ice-water bath.

(ii) US: samples were sonicated using a 450 digital sonifier (Branson Ultrasonics Corp., Danbury, CT), which is equipped with a temperature sensor (error ± 0.1 °C) and a tip of 13 mm diameter directly attached to a disruptor horn (20 kHz, 400 W full power) and immersed 2 cm in depth with respect to the liquid surface. Experiments were carried out at 50 and 70% of US wave amplitude for Lys-Glc model systems or at 70% for lactose solutions, taking 2 mL-aliquots at different sonication times (15, 30 and 60 min), which were immediately cooled in an ice-water. Throughout US treatments, temperature was kept

constant, depending on the test, at 25, 40 or 60 °C ± 2 °C, by immersing the beaker in an ice-water bath with control of temperature.

Part of the treated solutions was used directly for UV-absorbance, browning, and pH measurements, while the rest of samples were stored at -20 °C for 2-furoylmethyl-lysine (2-FM-Lys) and carbohydrate determinations.

2.4. Maillard reaction assessment

2.4.1. Initial steps of Maillard reaction: 2-Furoylmethyl-lysine determination

Samples (0.5 mL) were diluted to 3.5 mL with distilled water, and 1 mL was hydrolyzed at 110 °C for 23 h under inert conditions (helium) with 2.4 mL of 11.4 N HCl, resulting in a final concentration of 8 N HCl (Moreno, Molina, Olano, & López-Fandiño, 2003). After filtering through Whatman 40 filter paper, 500 μL of the hydrolysate was applied to a previously activated Sep-Pak C_{18} cartridge (Millipore Corp., Bedford, MA). 2-FM-Lys was eluted with 3 mL of 3 N HCl, and 50 μL was used for injection. Analysis was carried out via an ion-pair RP-HPLC method using a C_8 (Alltech furosine-dedicated; Alltech, Nicolasville, KY) column (250 x 4.6 mm i.d.) and a variable wavelength detector at 280 nm (LDC Analytical, SM 4000; LDC Analytical, Salem, NH). Operating conditions were as follows: column temperature, 35 °C; flow rate, 1.2 mL/min; solvent A, 0.4% HPLC grade acetic acid in double-distilled water; solvent B, 0.3% KCl in solvent A (Resmini, Pellegrino, & Batelli, 1991). Calibration was performed by using known concentrations (0.52 to 5.2 mg/L) of a pure standard of furosine (Neosystem Laboratories, Strasbourg, France). Data were expressed as milligrams per g of lysine. The analyses were carried out in duplicate and ~~average~~ relative standard deviation was minor to 10%.

157 *2.4.2. Intermediate and advanced steps of Maillard reaction*

158 The UV-absorbance and browning intensity of the aqueous solutions containing
159 glucose and lysine were measured spectrophotometrically at room temperature at 294 nm and
160 420 nm, respectively, using a microplate reader (Synergy-HT, BioTEK Instruments,
161 Winooski, VT). When necessary, appropriate dilutions were made in order to obtain an
162 absorbance of less than 1.5. The analyses were carried out in duplicate and ~~average~~ relative
163 standard deviation was minor to 10%.

164

165 *2.5. Lactose isomerization assessment*

166

167 The formation of lactulose, epilactose and galactose during heat treatment of lactose
168 solutions was determined by means of GC-FID analysis of the trimethylsilyl ethers of the
169 carbohydrate using an Agilent Technologies 7890A gas chromatograph (Wilmington, DE,
170 USA) equipped with a commercial fused silica capillary column SPB-17, bonded, crosslinked
171 phase (50% diphenyl/50% dimethylsiloxane; 30 m × 0.32mm i.d., 0.5 μm film thickness)
172 (Supelco, Bellefonte, PA, USA) (Montilla, Moreno, & Olano, 2005). Samples (15 μL) were
173 mixed with 0.5 mL of 0.4 mg/mL phenyl-β-D-glucoside (internal standard) in 70% ethanol.
174 The mixture was evaporated under vacuum at 40 °C and converted to trimethylsilyl
175 derivatives using *N*-trimethylsilylimidazole (Corzo-Martinez, Copovi, Olano, Moreno, &
176 Montilla, 2013).

177 To study the response factors relative to the internal standard, solutions containing
178 glucose, galactose, lactose and lactulose were prepared over the expected concentration range
179 in samples. For epilactose 1 was used as response factor. The amount of individual
180 carbohydrates present in the reaction mixtures were expressed as percentage by weight of the

total carbohydrate content. The analyses were carried out in duplicate and relative standard deviation was minor to 10%.

2.6. Statistical analysis

Data obtained from 2-FM-Lys determination and GC analysis of carbohydrates for Lys-Glc model systems and lactose solutions subjected to US and conventional heating treatments were statistically treated by using SPSS for Windows version 17.0. Univariate analysis of variance (ANOVA) (least squares means, Tukey's significant difference test) was used to determine the statistical differences between the treatments. Differences were considered significant when $p<0.01$.

3. Results

3.1. Effect of ultrasound on the Maillard reaction

To evaluate the initial steps of MR, the formation of 2-FM-Lys, derivative of Amadori compound, was chosen as sensitive parameter. This recognized indicator provides very valuable information since its early detection can prevent advanced stages of the MR in which important losses of nutritive value, mainly associated to the participation of essential amino acids in MR, are produced (Corzo-Martínez, Corzo, Villamiel, & del Castillo, 2012).

Fig. 1 illustrates the RP-HPLC chromatogram obtained after acid hydrolysis of the Lys-Glc model system treated by US at 70% US amplitude and 40 °C for 30 min. Similar profile (results not shown) was found for samples treated by conventional heating under the same conditions. As observed, two peaks with retention times of 17 and 18 min, were

detected. The most retained compound was coeluted with a commercial standard of ε-2-FM-Lys, suggesting that the less retained peak could correspond to α-2-FM-Lys, according to Moreno et al. (2003). Moreover, in agreement with these authors, the ratio between both compounds was approximately 4:1, respectively, indicating a higher reactivity of the ε-amino group (Ames, 1992). As no selective effect of US was observed for any of them, the sum of both species was considered for quantitation as 2-FM-Lys.

Table 1 lists the content of 2-FM-Lys in model systems of Lys-Glc (pH 7) subjected to US treatments at 50 and 70% of US wave amplitude and 25 and 40 °C and to conventional treatments carried out at the same temperature. As expected, the formation of 2-FM-Lys was higher at 40 than at 25 °C, since, as it is very well-known, MR widely depends on the reaction temperature.

At 25 °C no significant ($p>0.01$) differences were detected in the level of 2-FM-Lys in samples treated by US at 50% of wave amplitude and by conventional heating. However, when the amplitude of US oscillation was increased at 70%, a significant ($p<0.01$) acceleration of the initial steps of MR (at 30 min) was observed as compared to the other two treatments at 25 °C (UST 50% and CT), to subsequently decrease the level of 2-FM-Lys after 60 min of heating. This could be ascribed to the fact that, at this value of US amplitude, the intermediate and advanced stages could be also accelerated and the rate of Amadori compound decomposition might be higher than that of its formation.

Regarding 40 °C, the initial point (0 time) presented a certain content of 2-FM-Lys, since, as explained in Material and Methods, all samples (US and conventional treated) were subjected to identical heating rate to achieve 40 °C. At 50% of US amplitude, no significant differences were observed between US and conventional treated samples up to 30 min of reaction. However, the level of 2-FM-Lys was significantly ($p<0.01$) higher at 50 and 70% of

US amplitude for 60 and 15 min, respectively, as compared to model systems treated by conventional heating at identical temperature. Moreover, after 30 min of reaction at 40 °C and 70% of US amplitude, the amount of 2-FM-Lys also started to decrease due to the Amadori compound decomposition, as above indicated for treatments carried out at 25 °C. These results seem to indicate not only an acceleration of initial steps of MR by US but also a positive effect of them on the rate of more advanced stages of this reaction, this effect being also dependent of the US wave amplitude applied. A confirmation of these data was found by means of the evaluation of intermediate (A_{294}) and advanced stages (A_{420}) of the reaction (Fig. 2A and B, respectively). As observed, absorbance values increased with temperature and time of processing, and the highest values were detected in Lys-Glc samples treated by US at 70% of power. According to obtained results to respect furosine content and evaluation of intermediate and advanced stages the treatment at higher temperature (40 °C) with lower US ampitude (50%) wass efficient enough but more controllable in order to maintain/prolong early steps of MR. Guan et al. (2011) and Stanic-Vucinic et al. (2013) also observed, in model systems, an increase in the rate of intermediate and advances stages of MR with the increase of US power, but, to the best of our knowledge, no study of the initial stages has been previously done.

3.2. Effect of ultrasound on the formation and degradation of lactulose

The isomerization of lactose was followed by the formation of lactulose, epilactose and galactose. Fig. 3 shows the carbohydrate profile obtained after the treatment of lactose solutions by US at 70% of wave amplitude and 60 °C. Similar chromatogram (results not shown) was obtained by conventional treatment.

253 The quantitative data corresponding to the effect of US and conventional heating on
254 lactose isomerization are presented in Table 2. As observed, levels of lactose decreased with
255 increasing incubation time in samples subjected to both US and conventional heating
256 treatments. Moreover, in general, significantly ($p<0.01$) higher formation of lactulose was
257 found for lactose solutions at pH 10.6 (8 mM sodium hydroxide) as compared to the buffered
258 system at pH 10.0, probably due to the higher initial pH in the former since this reaction is
259 favoured with the increase of pH (Zokaee et al., 2002). However, after 30 minutes of reaction,
260 isomerization in buffered system was intensified more than in system with NaOH most likely
261 due to better maintenance of basic pH (data not shown). Similarly to the results observed for
262 the effect on MR, an initial amount of lactulose, epilactose and galactose was detected at 0
263 time since all the treatments (US and conventional) had the same heating rate.
264 With respect to the effect of US vs. conventional heating, in the buffered system (pH
265 10.0), an acceleration of isomerization reaction was observed as indicated by the significantly
266 ($p<0.01$) higher values of lactulose, epilactose and galactose in the case of samples subjected
267 to US treatments as compared to those of conventional ones under assayed conditions (70% of
268 US wave amplitude and 60 °C). In the case of sodium hydroxide system (pH 10.6), the
269 acceleration of lactose isomerization due to US treatment was not so clear as in the buffered
270 system, in spite of some significant differences in lactulose (at 15 min) and epilactose (at 15
271 and 30 min) values.
272
273 **4. Discussion**
274
275 As it is known, when US are applied in liquid systems, cavitation (formation and
276 violent collapse of bubbles) is the main involved phenomenon, although others such as

277 heating (specific absorption of acoustic energy), dynamic agitation and shear stresses and
278 microstreaming should be also considered (Floros & Liang, 1994). All these mechanisms
279 involved in the US treatment can induce physical and chemical effects and accelerate some
280 reactions (Soria & Villamiel, 2010). In fact, strong sheer forces generated during sonication
281 enable efficient mixing of solution and efficient heat/mass transfer contributing to increase the
282 rate of intermediate and advances stages of the MR (Stanic-Vucinic et al. 2013). In the present
283 work, this physical effect could be, therefore, related to the higher 2-FM-Lys and lactulose
284 concentration observed in the ultrasonicated model systems as compared to conventional
285 treated samples under the same temperature conditions. In addition, other considerations
286 should be also taken into account. Thus, US are well-known and efficient technology to
287 remove gas from solutions (Soria & Villamiel, 2010), and, on the other hand, it has been
288 described that dissolved oxygen has a significant effect on the formation of lactulose and
289 furosine in heated milks since, during the early stages of the MR and lactose isomerization, in
290 presence of oxygen, the double bond of enediols may be cleaved to produce carboxylic acids.
291 This effect is more pronounced for lactulose formation since the enediol precursor of Amadori
292 compound is less oxygen-sensitive and, at prolonged times of heating when considerable
293 proportion of oxygen is consumed, small differences in the content of furosine can be found
294 (Rada-Mendoza, Villamiel, & Olano, 2002). Thus, treatment with US could cause the removal
295 of oxygen, avoiding the oxidative cleavage of the enediols and increasing the amount of 2-
296 FM-Lys and lactulose formed as compared to samples treated by conventional heating.
297 The chemical effect should not be discarded in both reactions since during US
298 application in aqueous systems, water is cleaved into H• and •OH radicals, and with other
299 species present, various other radicals may be formed (Crum, 1995). As a consequence of this
300 mechanism more oxidation and lower 2-FM-Lys and lactulose could be expected in US

treated samples. However, according to the obtained data, the physical effect of mixing, efficient heat/mass transfer and removal of oxygen could be the predominant mechanism. Moreover, in the carbonate-bicarbonate buffer system used during lactose isomerization the formed radicals during US application that might favour the oxidation of intermediate compounds can be trapped by the ions carbonate and bicarbonate (Merouani, Hamdaoui, Saoudi, Chiha, & Petrier, 2010) and thus, increase the formation of lactulose.

5. Conclusions

On the basis of the obtained results, it is possible to say that the US assistance during MR and isomerization of lactose in heated model systems gives rise to higher levels of 2-FM-Lys and lactulose, respectively, than in the corresponding heating treatments carried out without US, although in the latter reaction a clear dependence of the type of system was observed. Although more research is needed to optimize the processes and to go more insight the involved mechanisms, the data here obtained point out the usefulness of US as complement of heating to promote the formation of functional ingredients by MR and lactose isomerization.

Abbreviations Used:

CT: conventional treatment

Lys-Glu: lysine-glucose

MR: Maillard reaction

US: ultrasound

UST: ultrasound treatment

2-FM-Lys: 2-furoylmethyl-lysine

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FIGURE CAPTIONS

Fig. 1. RP-HPLC-UV chromatogram of 2-FM-Lys in acid hydrolysate of the lysine-glucose model system treated by US (70% amplitude) after 30 min at 40 °C. Peak 1: α-2-furoylmethyl-lysine and Peak 2: ε-2-furoylmethyl-lysine (furosine).

Fig. 2. Evolution of the absorbance at 294 (A) and 420 nm (B), as indicators of intermediate and advanced stages of MR, in lysine-glucose model systems after 15, 30 and 60 min of ultrasound (UST) and conventional (CT) heating treatments at 25 and 40 ºC and 50 and 70% of wave amplitude in the case of US. Data are average of two independent experiments ± standard deviation of the mean.

Fig. 3. Gas chromatographic profile of the trimethylsilyl derivatives of carbohydrates present in 10% lactose solutions subjected to US heating treatment at 70% of amplitude and 60 °C during 60 min.

Figure 1.

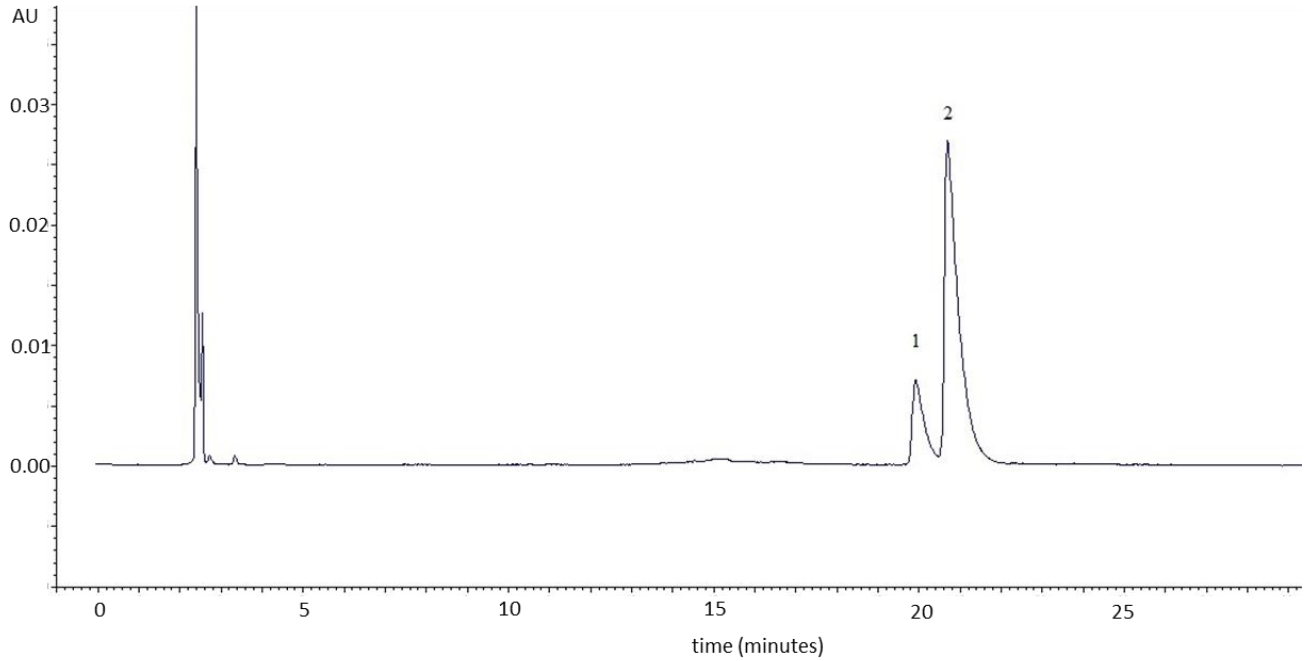

Figure 2.

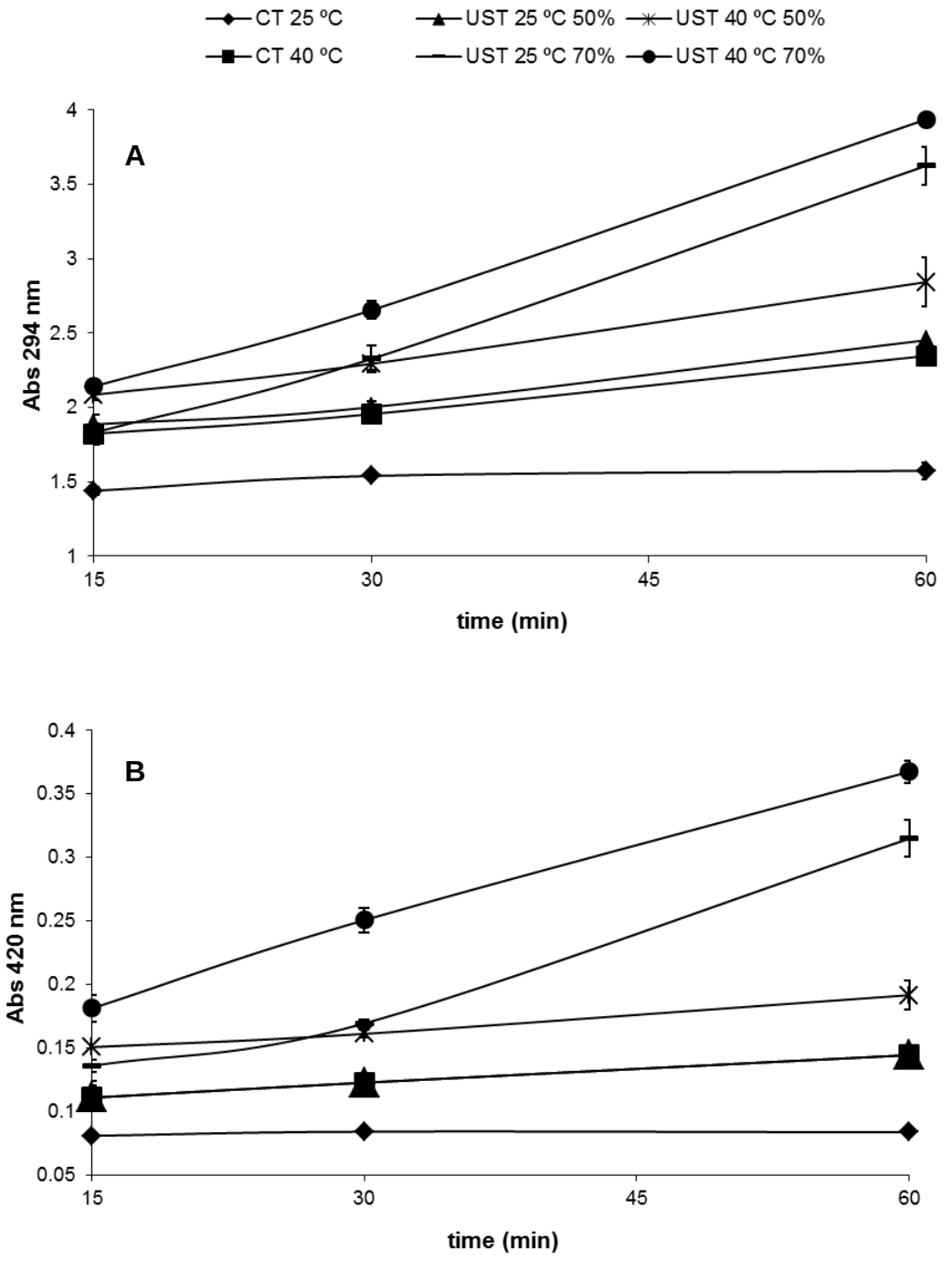
CT 25 ºC
UST 25 ºC 50%
UST 40 ºC 50%
CT 40 ºC
UST 25 ºC 70%
UST 40 ºC 70%
A
Abs 294 nm
time (min)
B
Abs 420 nm
time (min)

Figure 3.

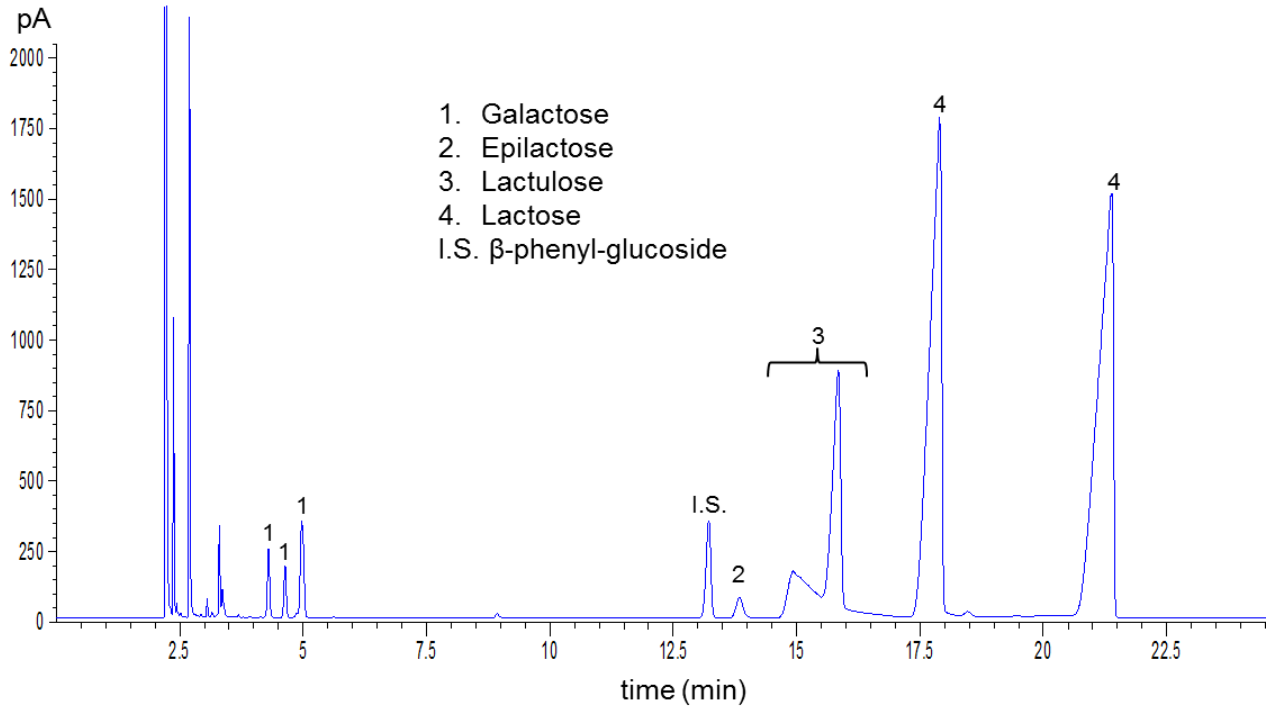
pA
2000
1750
1500
1250
1000
750
500
250
0
1. Galactose
2. Epilactose
3. Lactulose
4. Lactose
I.S. β-phenyl-glucoside
1
1
1
I.S.
2
3
4
4
2.5
5
7.5
10
12.5
15
17.5
20
22.5
time (min)

Table 1. Contents of 2-furoylmethyl-lysine (milligrams per g of lysine ± SD) obtained after acid hydrolysis of lysine-glucose model systems subjected to different ultrasound (UST) and conventional (CT) heating treatments at pH 7.0. Data correspond to the sum of α- and ε-2-furoylmethyl-lysine.

Treatment			Time (min)[1]			
	T (°C)	Amplitude (%)	0	15	30	60
CT	25		0.00 ± 0[a]	120.69 ± 3.98 [a]	127.22 ± 5.18 [a]	127.99 ± 7.06 [a]
CT	40		49.67 ± 2.11 [b]	138.32 ± 12.76 [b]	137.08 ± 5.96 [ab]	142.68 ± 4.92 [b]
UST	25	50	0.00 ± 0 [a]	125.39 ± 9.61 [ab]	129.69 ± 7.82 [a]	135.23 ± 6.52 [ab]
UST	25	70	0.00 ± 0 [a]	134.81 ± 2.56 [ab]	145.07 ± 3.82 [b]	128.82 ± 7.97 [a]
UST	40	50	49.67 ± 2.11 [b]	138.09 ± 1.12 [ab]	142.94 ± 12.78 [b]	159.59 ± 9.64 [c]
UST	40	70	49.67 ± 2.11 [b]	165.18 ± 15.73 [c]	126.69 ± 10.72 [a]	134.72 ± 13.38 [ab]

[a-c] Different case letters indicate statistically significant ($p<0.01$) differences at the same period of time.

Table 2. Degradation of lactose and formation of galactose, epilactose, and lactulose (average percentage of total carbohydrate content ± SD) during ultrasound (UST, 70% amplitude) and conventional (CT) heating treatments at 60 °C of 10% lactose solutions at pH 10.0 (buffer) and pH 10.6 (8 mM NaOH).

Treatment		Time (min)[1]			
	Basic media	0	15	30	60
Galactose					
CT	Buffer	0.77 ± 0.22 [a*]	1.45 ± 0.10 [a]	2.07 ± 0.13 [a]	3.62 ± 0.29 [a]
CT	8 mM NaOH	1.06 ± 0.06 [b]	1.49 ± 0.10 [a]	2.13 ± 0.04 [a]	3.21 ± 0.26 [ab]
UST	Buffer	0.77 ± 0.22 [a]	2.00 ± 0.15 [b]	3.41 ± 0.37 [b]	5.82 ± 0.48 [c]
UST	8 mM NaOH	1.06 ± 0.06 [b]	1.54 ± 0.12 [a]	2.30 ± 0.18 [a]	2.77 ± 0.61b[c]
Epilactose					
CT	Buffer	0.21 ± 0.04 [a]	0.44 ± 0.02 [a]	0.65 ± 0.02 [a]	1.10 ± 0.04 [a]
CT	8 mM NaOH	0.33 ± 0.03 [b]	1.09 ± 0.01 [b]	1.45 ± 0.02 [b]	1.92 ± 0.08 [b]
UST	Buffer	0.21 ± 0.04 [a]	0.67 ± 0.04 [c]	1.02 ± 0.02 [c]	1.71 ± 0.08 [c]
UST	8 mM NaOH	0.33 ± 0.03 [b]	1.24 ± 0.05 [d]	1.67 ± 0.09 [d]	1.77 ± 0.15 [bc]
Lactulose					
CT	Buffer	3.90 ± 0.50 [a]	9.26 ± 1.04 [a]	12.25 ± 0.28 [a]	18.53 ± 0.34 [a]
CT	8 mM NaOH	8.58 ± 0.79 [b]	17.79 ± 0.17 [b]	22.04 ± 0.22 [b]	25.95 ± 1.13 [b]
UST	Buffer	3.90 ± 0.50 [a]	11.35 ± 0.30 [c]	16.48 ± 0.11 [c]	22.72 ± 1.25 [c]
UST	8 mM NaOH	8.58 ± 0.79 [b]	19.34 ± 0.26 [d]	22.96 ± 1.03 [b]	23.94 ± 1.01 [bc]
Lactose					
CT	Buffer	95.11 ± 0.77 [a]	88.90 ± 1.25 [a]	85.09 ± 0.38 [a]	76.72 ± 0.42 [a]
CT	8 mM NaOH	90.13 ± 0.90 [b]	79.62 ± 0.14 [b]	74.37 ± 0.2 [b]	68.92 ± 1.25 [b]
UST	Buffer	95.11 ± 0.77 [a]	86.08 ± 0.40 [c]	79.07 ± 0.29 [c]	70.11 ± 1.77 [b]
UST	8 mM NaOH	90.13 ± 0.90 [b]	77.90 ± 0.32 [d]	73.05 ± 1.20 [b]	71.51 ± 1.72 [b]

[a-d] Different case letters indicate statistically significant ($p<0.01$) differences at the same period of time